In vitro assembly of cytochrome oxidase from separately accumulated subunits: a reconsideration

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1. INTRODUCTION

A most obscure aspect of mitochondrial biogenesis is the mechanism of assembly of the enzymic complexes in the mitochondrial inner membrane. Early works from the laboratory of Green [1–3] have shown that mixing of concentrated solutions of respiratory complexes gives rise to vesicles which can perform the functions of an uncoupled respiratory chain. The latter cannot be reconstituted by mixing already diluted solutions of the complexes. Such solutions were supposed [3] to contain vesicles carrying a single species of the complexes, and exchange of complexes between different vesicles to form mixed systems was held to be impossible.

Of special interest in this connexion is a report by Chandrasekaran et al. [4] on the assembly of cytochrome *c* oxidase in yeast mitochondria. This enzyme of the mitochondrial inner membrane is believed to comprise seven subunits, three of which are made on mitochondrial and four on cytoplasmic ribosomes [5]. The authors [4] claimed that catalytically active cytochrome oxidase was assembled from mitochondrial and cytoplasmic subunits upon mixing of two preparations of mitochondria containing an excess of either mitochondrial or cytoplasmic subunits accumulated by incubating yeast spheroplasts with inhibitors of either cytoplasmic (cycloheximide, CHI) or mitochondrial (chloramphenicol, CAP) protein synthesis, respectively. In obvious contrast to the above concept [3], this implied an exchange of cytochrome-oxidase subunits between two membrane systems.

The experimental protocol of [4] was not without fault. In particular, the assembly of the complex was inferred solely from the increase in cytochrome oxidase activity in the mixed preparation. To do away with ambiguity, one should have tried to obtain data on the content of cytochromes aa_3 as well as to study the enzymic changes in more detail.

Such a check reported here shows that the suggestion [4] about the assembly of cytochrome oxidase from subunits harboured by different mitochondrial membranes can hardly hold true.

2. MATERIALS AND METHODS

Saccharomyces cerevisiae wild-type diploids were grown aerobically [6] in the following medium: 10 g glucose, 1 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 2 g yeast extract (Serva, Heidelberg) per litre. After the beginning of glucose derepression (judged by a distinct increase in cell respiration by 12 h of growth) the batches were further incubated in the same conditions (control) or in the presence of CAP (4 g/litre) or CHI (100 mg/litre) as proposed in [4].

Cell respiration was measured amperometrically in 50 mM potassium phosphate pH 7.4 with 0.25% glucose at 30°C with a Clark oxygen electrode and a Radiometer PO-4 polarograph.

Yeast cell were disrupted by the method of Tzagoloff [7] in 0.6 M sorbitol, 20 mM Tris–maleate (pH 6.7 at 4°C), 1 mM EDTA. Debris was removed at 1000 × g_{max} and mitochondria were pelleted at 20 000 × g for 20 min, washed and resuspended in the same medium.

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Cytochrome oxidase activities of the mitochondrial fractions and their mixtures were assayed (1) as proposed in [4], i.e., without any detergent treatment, and (2) after maximal activation achieved by freezing–thawing the membranes twice in the presence of a non-ionic detergent (0.06% Lubrol WX) and including the detergent in the assay mixture [8].

Room-temperature difference spectra (reduced minus oxidized) of mitochondria were recorded with an Aminco DW-2a UV/VIS spectrophotometer.

3. RESULTS AND DISCUSSION

Yeast undergoing glucose derepression were incubated for 4 h with inhibitors of either mitochondrial (CAP) or cytoplasmic (CHI) translation. As shown in tables 1 and 3, either inhibitor suppressed the development of cell respiration and the increase in cytochrome aa_3 content and in cytochrome oxidase activity. Mitochondrial fractions obtained from cells treated with CAP or CHI were then mixed in equal amounts (on the protein basis).

Enzymic measurements performed in conditions used by Chandrasekaran et al. [4] showed that cytochrome oxidase activities are rather low and that the activity of the mixed preparation exceeds the arithmetic average of the activities of initial fractions (table 2). In other words, these experiments apparently reproduced the 'activation' phenomenon which has been ascribed [4] to 'complementation' between enzyme subunits separately accumulated in the two membrane preparations.

However, subsequent experiments demonstrated that cytochrome oxidase activity markedly increases upon solubilization of the mitochondrial membranes (see Materials and Methods), and may exceed those measured according to [4] more than 3-fold (cf. tables 2 and 3). But the most important point is that the mixture of mitochondria from CHI- and CAP-treated cells displays activity which is the arithmetic mean of the activities of the two initial fractions.

It is noteworthy that the comparison of the cytochrome aa_3 contents and cytochrome oxidase activities in the non-activated mitochondria reveals no correlation. On the other hand, the maximal

Table 2

Mitochondrial cytochrome oxidase activities assayed according to Chandrasekaran et al. [4].

	μmol cyt c . min $^{-1}$. mg $^{-1}$
Blank	3.04
CHI-preparation	4.98
CAP-preparation	2.27
Mixed CHI/CAP preparation	5.05
Expected average	3.63

The assay buffer contained 17 μM ferrocytochrome c.

Table 1

Effects of CHI and CAP on the development of cell respiration during glucose derepression in yeast

Culture growth (h)	Sample	Respiration, (ng-atom of O . min $^{-1}$. mg dry wt $^{-1}$)	Increase (%)
12	Zero (inhibitors added at this moment)	104	–
16	Blank	251	100
16	CHI	122	17
16	CAP	180	51

After 12 h of aerobic growth the culture was divided into three batches, one of which was further grown without additions (blank) and the others with either CHI (100 mg/litre) or CAP (4 g/litre) for 4 h.

Table 3

Cytochrome aa_3 contents in mitochondrial preparations and 'maximal' cytochrome oxidase activities of solubilized membranes

	nmol $aa_3 \cdot mg^{-1}$	μmol cyt $c \cdot min^{-1} \cdot mg^{-1}$
Blank	0.392	10.01
CHI-preparation	0.326	8.19
CAP-preparation	0.269	6.82
Mixed CHI/CAP preparation	0.296	7.33
Expected average	0.297	7.50

Mitochondria were solubilized as described in Materials and Methods, and enzymic assays were performed in 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 0.06% Lubrol WX, with 17 μM ferrocytochrome c as substrate.

activities are in exact correspondence to the contents of cytochrome aa_3 (table 3), both characteristics being simply averaged on mixing of CHI- and CAP-treated preparations.

Thus it can be concluded that, at least in our hands, the 'increment' of cytochrome oxidase activity in the mixed mitochondria, which is similar to that reported in [4], is only due to the inadequacy of the enzymic assay. Therefore we have yet no serious grounds for believing that on mixing of two mitochondrial preparations, one of which contains excess mitochondrial subunits and the other cytoplasmic, exchange of subunits or membrane fusion may take place so as to form the cytochrome oxidase complex.

Taking into account this negative result, one should still bear in mind that a principal road to elucidating the mechanism of cytochrome oxidase assembly lies through separately studying the stages of synthesis, processing, and transport of subunits, as well as their integration into the catalytic complex. While the first three steps are at present under intensive investigation (see, e.g., [9–11]), the last one is still neglected. Yet, the problem of the assembly of the mitochondrial membrane complexes obviously can not be solved without energetic research along this line.

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